Isolation and Characterization of Small Molecular weight Pharmacophores from Marine Gastropods as Defense Metabolites against Oxidative Stress Induced Diseases

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The present investigation entitled "*Isolation and characterization of small molecular weight pharmacophores from marine gastropods as defense metabolites against oxidative stress induced diseases*" has been discussed under eight chapters. The primary sections discussed the Acknowledgements, Abbreviations, Contents, Abstract, list of tables and list of figures. The chapters are divided into eight followed by references and the list of publications, which were detailed as follows:

CHAPTER 1: INTRODUCTION

This is an introductory chapter, which was dealt with a brief introduction on the importance and significance of bioactive properties from marine gastropods, especially Family Muricidae and Babylonidae with their antioxidant, antidiabetic and anti-inflammatory potentials.

The marine gastropods have been the subject of a significant amount of biological and chemical research over the past five decades worldwide. In many cultures, shelled gastropods have been regarded as a delicacy or healthy food, and they also feature in a range of traditional natural remedies. In India, the gastropods are particularly exploited by fisherman for mere shell craft industries, although meager studies are available to document the presence of bioactive metabolites in these species. Marine ecosystems of the Indian subcontinent support the unexplored diversity of life, and represent a vast untapped resource of gastropods with potential benefits in pharmaceuticals and for the discovery of potential chemotherapeutic agents. The present study is focused to isolate and characterize bioactive compounds selected marine gastropods for use against oxidants along with the oxidative stress induced diseases, such as type-2 diabetes and inflammation.

Based on this, the objectives of the thesis are as follows:

- To evaluate tropical marine gastropod mollusks (*Chicoreus ramosus* and *Babylonia spirata*) from marine ecosystem for nutritional quality and selective bioactivities against oxidants, type-2 diabetes and inflammation.
- To isolate the small molecular weight bioactive leads by extensive chromatographic procedures including preparatory high pressure liquid chromatography.
- Validation of pharmacological properties of the purified compounds with regard to anti-inflammatory, antioxidative and antidiabetic activities using selective assays (inhibition of α-amylase/α-glucosidase/5-lipoxidase (LOX-5) activities, 2,2-diphenyl-1-picrylhydrazyl (DPPH) /2,2'-azino-bis-3-ethylbenzothiozoline-6-sulfonic acid ($ABTS^{+}$)/etc).

- ❖ To characterize the purified bioactive compounds by detailed spectroscopy, such as nuclear magnetic resonance (NMR), Fourier transform infrared (FTIR), and mass spectral analyses.
- ❖ To predict the target biological properties by structure-activity relationship and generation of database of potential leads with selective anti-inflammatory, antioxidative, and antidiabetic activities as tool box to combat oxidative stress induced diseases.

Based upon the above objectives, the present Thesis is classified into eight chapters. The importances of this study on the bioactive potentials of the selected marine gastropods, along with objectives were discussed in the introductory chapter (Chapter 1). The detailed review of the studies was based on the significance of marine gastropods as antioxidative and anti-inflammatory bioactive source along with the characterized bioactive compounds with their medicinal values were envisaged in the Chapter 2. Nutritional profiling of the selected marine gastropods are included in Chapter 3. Chapter 4 described the in vitro evaluation of the antioxidant, antidiabetic and anti-inflammatory potentials of the organic extracts of the selected marine gastropods *Chicoreus ramosus* and *Babylonia spirata*. The isolation and characterization of secondary metabolites responsible for antioxidant, antidiabetic and anti-inflammatory activities from *C. ramosus* and *B. spirata* have been described in Chapter 4 and Chapter 5, respectively. The isolation and characterization of the polysaccharides responsible for anti-inflammatory activities from *Babylonia spirata* were described in Chapter 7. The summary and conclusion of the work and major research findings from the current study were detailed in Chapter 8.

CHAPTER 2: REVIEW OF LITERATURE

The importance and background marine gastropods as potential sources of bioactive compounds and their utilities were described in this Chapter. Marine gastropods have been used in folklore medicines and extracts from gastropod species have proven activity against human, animal and plant pathogens. Marine gastropods are biochemically unique, producing wide array of natural products with unique bioactivity. They possess active metabolites with some novel chemical structures, which belong to diverse chemical classes, such as alkaloids, phenols, steroids, terpenoids etc. However, the scientific information about the biological effects of marine gastropods and active substances is scarce and poorly documented. Many of the compounds isolated from marine gastropods showed pharmacological activities and have been helpful for the invention and discovery of drugs, primarily for deadly diseases, such as

cancer, acquired immuno-deficiency syndrome (AIDS), arthritis, etc. A detailed literature survey on the secondary metabolites isolated and characterized from the marine gastropods with antioxidant, and anti-inflammatory functionalities were reported in this Chapter.

CHAPTER 3: NUTRITIONAL QUALITIES OF BRANCHED MUREX *CHICOREUS RAMOSUS* (FAMILY MURICIDAE) AND THE BUCCINID *BABYLONIA SPIRATA* (FAMILY) BABYLONIDAE

This chapter presents a detailed nutritional profiling of the marine gastropods *C. ramosus* and *B. spirata.*

The sample collection and pre-treatment and the analysis of various nutritional parameters such as total lipid content and fatty-acid profile; total protein and amino acid composition; total carbohydrate content; mineral content; fat Soluble (A, D, E, K) and water soluble (C) vitamins and total cholesterol content of the candidate gastropods by established methods are detailed in this chapter. The study demonstrated the low-value species *C. ramosus* as well as the commercial gastropod *B. spirata* as a rich storehouse of key nutritional elements required by the human nutrition and metabolic pool. These marine gastropods were found to be significantly valuable sources of protein with the more prominent content of essential/non–essential amino acid ratio. More noteworthy levels of C_{20-22} long chain *n*–3 polyunsaturated fatty acids, and greater *n*–3/*n*–6 fatty acid proportion (~0.1) demonstrated that the gastropods *B. spirata* and *C. ramosus* are good sources of well–balanced diets, and can counterbalance the increased consumption of vegetable oils rich in *n*-6 fatty acids. The ideal atherogenicity/ thrombogenicity indices (≤1.0), hypocholesterolemic /hypercholesterolemic ratio and fatty acid /amino acid based health markers in the edible portions qualified these specie as potential health food. The candidate gastropods were also found to be the valuable source of numerous critical micro and macro minerals and vitamins, which are fundamental for the metabolic functioning of the body. *B. spirata* and *C. ramosus* can be considered as a new source of important health food, and can be used in formulating various functional food ingredients for pharmaceutical industries.

. **CHAPTER 4: BIOACTIVE POTENTIALS OF THE CRUDE EXTRACTS OF THE STUDIED MARINE GASTROPODS WITH RESPECT TO ANTI-OXIDATIVE, ANTI-DIABETIC AND ANTI-INFLAMMATORY PROPERTIES**

4.1.1. Antioxidant activities of solvent extracts from selected marine gastropods

This section detailed the antioxidant activities of chloroform ($CHCl_3$) and MeOH-EtOAc from the extract of each sample by using different *in vitro* antioxidant assays. The *in vitro* assays included radical scavenging activities by using oxidants, such as 1,1-diphenyl-2-

picryl-hydrazil ($DPPH^{\cdot}$), 2,2′-azino-bis-3-ethylbenzothiozoline-6-sulfonic acid ($ABTS^{+}$), and ferrous ion (Fe^{2+} ion) along with thiobarbituric acid reactive species (TBARS) formation inhibitory activity and total phenolic content.

Among the two different solvent extracts obtained from the selected marine gastropods *C. ramosus* and *B. spirata*, the EtOAc-MeOH fraction acquired from *B. spirata* exhibited significantly ($P<0.05$) greater DPPH radical scavenging activity (IC_{50} 1.91 mg/mL) followed by the EtOAc-MeOH fraction from *C. ramosus* (IC_{50} 2.41 mg/mL) than other solvent fractions (IC_{50} >2.5 mg/mL). Among these fractions, EtOAc-MeOH fraction from *B. spirata* recorded significantly greater ABTS radical scavenging activity (IC_{50} 0.84 mg/mL) followed by that of EtOAc fraction of *C. ramosus* (IC_{50} ~2.0 mg/mL) ($p<0.05$) (Table 1). The ferrous ion (Fe^{2+} ion) chelating activity was also greater for EtOAc fraction of *B. spirata* (IC_{50} 1.22 mg/mL) followed by its $CHCl_3$ fraction (IC_{50} ~1.70 mg/mL), whereas the TBARS formation inhibitory activity was also found to be greater for the EtOAc fraction obtained from *B. spirata* (IC_{50} 2.50 mg/mL) followed by its $CHCl_3$ fraction (IC_{50} ~3.40 mg/mL). Consequently, among all solvent fractions, the ethyl acetate fractions obtained from these two species exhibited potent activities than their corresponding $CHCl_3$ fractions.

Table 4.1. Antioxidative, anti inflammatory and anti-diabetic activities of the solvent fractions obtained from the selected marine gastropods *C. ramosus* and *B. spirata*

Bioactivities†	**IC_{90} (mg/mL)**			
	C. ramosus		***B. spirata***	
Antioxidative activities	**EtOAc-MeOH**	**$CHCl_3$**	**EtOAc-MeOH**	**$CHCl_3$**
DPPH. scavenging	$2.41^{b} \pm 0.56$	$5.65^{c} \pm 0.16$	$1.91^{a} \pm 0.07$	$4.79^{c} \pm 0.04$
$ABTS^{+\cdot}$ scavenging	$2.01^{b} \pm 0.26$	$5.23^{d} \pm 0.03$	$0.84^{a} \pm 0.04$	$3.60^{c} \pm 0.06$
Fe^{+2}	$1.83^{b} \pm 0.21$	$2.90^{c} \pm 0.05$	$1.22^{a} \pm 0.01$	$1.70^{b} \pm 0.02$
TBARS formation inhibitory	$4.31^{c} \pm 0.15$	$5.10^{d} \pm 0.05$	$2.50^{a} \pm 0.16$	$3.40^{b} \pm 0.10$
Total phenolic Content	$46.51^{b} \pm 1.03$	$20.16^{c} \pm 1.48$	$73.62^{a} \pm 1.26$	$55.60^{b} \pm 0.91$
Anti-inflammatory activities				
5-LOX inhibition	$1.74^{a} \pm 0.12$	$1.93^{b} \pm 0.08$	$1.97^{b} \pm 0.02$	$2.36^{c} \pm 0.02$
Anti-diabetic activities				
α-Amylase	$1.06^{a} \pm 0.01$	$2.13^{b} \pm 0.02$	$2.32^{b} \pm 0.05$	$3.85^{c} \pm 0.04$
α-Glucosidase	$2.25^{a} \pm 0.06$	$3.18^{b} \pm 0.16$	$4.5^{c} \pm 0.02$	$7.38^{d} \pm 0.03$

† The bioactivities were expressed as IC_{90} values (mg/mL). The samples were analyzed in triplicate (n=3) and expressed as a mean ± standard deviation. Means followed by the different superscripts (a–d) within the same row indicate significant differences ($P < 0.05$).

4.1.2. *In vitro* antidiabetic and anti-inflammatory activities of solvent extracts from selected marine gastropods

The different solvent fractions obtained from the selected marine gastropods (*C. ramosus* and *B. spirata*) were studied for their anti-inflammatory properties with regard to 5-LOX inhibition assays (Table 3.1). The anti-inflammatory results for EtOAc-MeOH fraction obtained from *C. ramosus* exhibited significantly greater activities (5-LOX IC_{90} 1.74 mg/mL) compared with the other solvent fractions (IC_{90}> 1.9 mg/mL). The EtOAc-MeOH fraction of *B. spirata* showed higher inhibitory activity towards 5-LOX (IC_{90} 1.97 mg/mL) followed by that of its chloroform fraction (IC_{90} 2.36 mg/mL).

4.1.3. Correlation analysis

One way analysis of variance (ANOVA) was carried out with the Statistical Program for Social Sciences 13.0 (SPSS, ver. 13.0, SPSS Inc., Quarry Bay, HK) to assess for any significant differences between the means. The significant differences were represented as $P<0.05$ and the values were given as mean of triplicate standard deviation. The mean variance in the data set was detected using principal component analysis (PCA). This section explained the relations between various bioactivities based upon statistical evaluations.

4.1.4. Spectroscopic labelling of the functional groups of solvent fractions of selected gastropods

The labeling of protons associated with various magnetic environments of the distinguished functional groups in the different solvent extracts were analyzed by proton spectroscopy. Detailed comparisons of proton integral values at different regions were plotted for chloroform and EtOAc-MeOH extracts prepared from the title species *C. ramosus* and *Babylonia spirata*

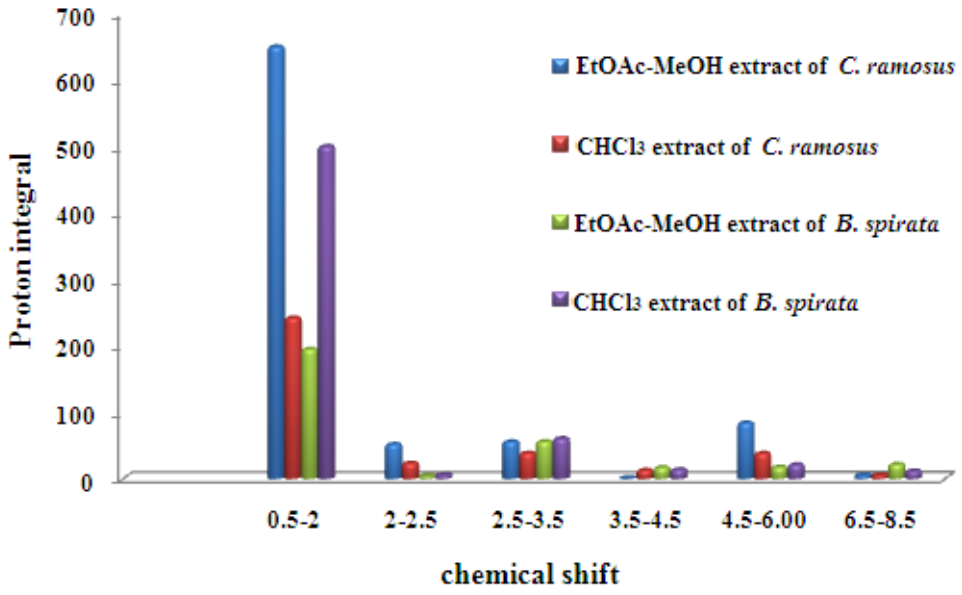


Fig. 2. Type and integral values of protons obtained from the ^{1}H-NMR of the solvent extracts of the title species *C. ramosus* and *B. spirata.*

CHAPTER 5: ISOLATION AND CHARACTERIZATION OF SECONDARY METABOLITES FROM BABYLONIA SPIRATA

5.1. Materials and Methods

This section described the instruments used for the present study along with the reagents and solvents used.

5.1.1. Sample Preparation

The live specimens of the gastropod *B. spirata* were collected from the landing centre at the south-west coastline of peninsular India (Lat, 8° 48’ 0N; Long, 78° 09’ 0E), and were shifted to the laboratory under deep-freezed conditions. The edible parts were carefully removed from the shells and rinsed with distilled water to remove associated debris.

The samples of *B. spirata* (5.50 kg) were cut into small pieces, macerated and freeze dried by utilizing a laboratory freeze-drier (Martin Christ alpha 1-4 LDplus, Martin Christ, Germany) yielding lyophilized powder of 1400 g. The lyophilized powder (500 g) was sonicated with solvent chloroform (500 mL X 2) and EtOAc-MeOH (1:1 v/v, 2 x 500 mL), for 2 h under an inert atmosphere of N_2 before being sifted through Whatman filter paper No. 1 to acquire the clarified filtrates (1 L). The clarified filtrates were dried through Na_2SO_4 (100 g), and evaporated (45^{o}C) by means of a rotary evaporator (Heidolph Instruments GmbH and Co., Schwabach, Germany) under vacuum to get orange yellow viscous residues. The yields based on wet material were 25.45 percent.

5.1.2. Chromatographic Purification and Spectral Analysis of Pure Compounds from *Babylonia spirata*

The crude EtOAc-MeOH fraction (50 g) of *B. spirata* was adsorbed on silica gel (60-120 mesh, 10 g), and loaded into a glass column (5 cm x 150 cm) packed with silica gel (60–120 mesh sized, 100 g). An initial elution of the column was carried out with the solvent *n*-hexane to remove the pigmented and waxy material. The eluent polarity (*n*-hexane/EtOAc 9:1 to 2:8, v/v) was gradually increased to provide 13 fractions of 100 mL each, which were reduced to 4 groups (A-D) based on TLC (*n*-hexane/EtOAc, 9:1, v/v) analysis. The key fraction A (50.0 mg) was subjected to column chromatography on silica gel (60-120 mesh) to afford 25 fractions (50 mL each) which were pooled to six groups (A1-A6) based on analytical TLC. The subfraction A-5 on consequent purification by open column using silica gel as stationary phase and the polarity of the eluent progressively increased using *n*-hexane-EtOAc, 100% EtOAc, and thereafter to EtOAc-MeOH, until 100 % MeOH afforded mixtures pooled to 12 groups (A5-1-A5-12) based on TLC pattern. The fraction A-5-7 was fractionated over preparatory TLC on silica gel GF_{254} using *n*-hexane/EtOAc (1:1, v/v) to afford BS-1. The compound, BS-1 was tested for its purity using TLC {*n*-hexane: EtOAc (7:3, v/v)} and RPC18 HPLC (MeOH/MeCN, 4:1) methodologies. The major fraction C on repetitive column chromatographic purifications yielded the sub fraction C-4-3 which upon fractionation by flash chromatography (Biotage, 230-400 mesh, 12 g; Sweden, Biotage No. 25+M 0489-1) at UV collection wavelength of 258 nm using *n*-hexane/EtOAc and EtOAc/MeOH mixtures of increasing polarity afforded BS-2. The homogeneity of the compound was substantiated by RP-C_{18}-HPLC (MeOH/ACN, 3:2 v/v, R_t 3.7 min) analysis. The pooled fraction B6 obtained by the fractionation of the major fraction B was flash chromatographed (Biotage, Silica gel 230-400 mesh, 12 g; Sweden, Biotage No. 25+M 0489-1) at UV collection wavelength of 258 nm using step gradient of *n*-hexane/EtOAc to afford a mixture B-6-3-5 which was further subjected to a Waters X-bridge semi-preparative column under the conditions of RP-C_{18}-HPLC {Waters X-bridge 150 x 2 mm; MeOH/ACN [5:95] to [95:5] linear gradient} which showed two major peaks for the compounds denoted BS-3 and BS-4.

5.1.3. Bioactive Potentials of Secondary Metabolites Isolated from *Babylonia spirata*

The bioactive potentials of ten pure compounds isolated from *Babylonia spirata* were checked by various *in vitro* antioxidants (DPPH and $ABTS^{+}$) antidiabetic and anti-inflammatory (5-LOX) assays and their bioactivities were expressed as IC_{50} values (in mg/mL).

5.1.4. Statistical analysis

One way analysis of variance (ANOVA) was carried out with the Statistical Program for Social Sciences 13.0 (SPSS, ver. 13.0, SPSS Inc., Quarry Bay, HK) to assess for any significant differences between the means. The significant differences were represented as $P<0.05$ and the values were given as mean of triplicate standard deviation. Statistical analyses were based upon different bioactivities of the pure compounds *vis-à-vis* standards, with regard to the antioxidant and anti-inflammatory activities.

5.2. Results and Discussion

5.2.1. Chromatographic Purification and Spectral Analysis of Secondary Metabolites from *B. spirata*

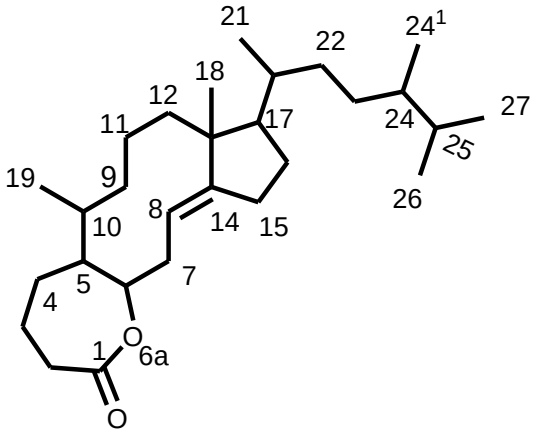
Repeated chromatographic purifications of the solvent fractions obtained from *B. spirata* led to the isolation of four new secondary bioactive metabolites. The structures were elucidated and confirmed by extensive analysis of the ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY and mass spectra along with FTIR analyses.

5.2.1.1. Structural Characterization of Compound BS-1

The compound BS-1 was isolated as white solid upon chromatographic purifications. The molecular ion peak observed at *m/z* 416 along with extensive spectroscopic analyses were suggested its molecular formula as $C_{28}H_{48}O_2$. The double bond equivalencies were calculated as five, which included two unsaturations and three ring skeletons. The ^{13}C NMR specified the incidence of 28 carbon signals. The DEPT spectrum together with ^{1}H and ^{13}C were exhibited resonances due to six methyls, eleven methylenes, eight methine carbon atoms and the remaining three signals were attributable to quaternary carbon atoms. The six methyl signals were appeared at δ 11.3, 18.7, 19.3, 14.1, 22.8 and 22.7. The signal due to trisubstituted olefinic group was appeared at δ 122.6 and 139.7 (-<u>C</u>H=<u>C</u><) in the ^{13}C NMR spectrum. The deshielded signal at δ 173.0 was indicated the presence of an ester carbonyl functionality. The basic skeleton of titled compound was found to be an ergostane with lactonic functionality in the A ring along with bond cleavages in the A-B rings and the nomenclature of the derived steroid was ascertained by the previous literature reports as 1,10:8,9-disecoergosta-8-en-*A*-*homo*-6a-oxa-1-one.

Compound BS-1

1,10:8,9-Disecoergosta-8-en-A-homo-6a-oxa-1-one



Sample yield	0.051 % based on dry weight
Physical description	White amorphous solid
Molecular formula	$C_{22}H_{40}O_6$
Molecular weight	416.3658

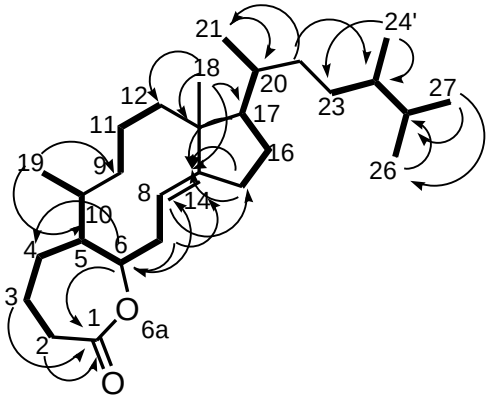


Fig. 5.2.1.1. Key ^{1}H-^{1}H COSY and HMBC correlations of 1,10: 8,9-disecoergosta-8-en-*A-homo*-6a-oxa-1-one (**BS-1**). The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; the HMBC couplings are indicated as single barbed arrow.

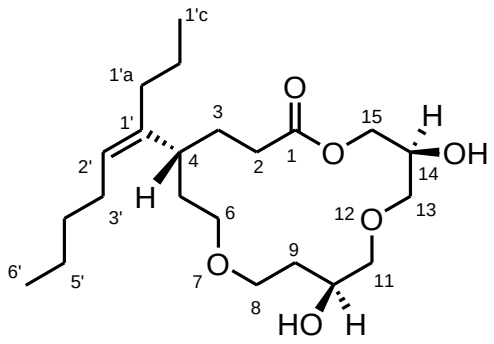
5.2.1. 2. Structural Characterization of Compound BS-2

The purified compound BS-2 was obtained as a pale yellow liquid. The molecular ion peak appeared at *m/z* 400 $[M]^+$ {HR (ESI) MS *m/z* found 401.2899 $[M+H]^+$, calcd. for $C_{22}H_{41}O_6$ 401.2903} in accordance with its one-dimensional (1D) and two-dimensional (2D) NMR data, which described the elemental composition of the compound as $C_{22}H_{40}O_6$. The ^{13}C-NMR and DEPT spectra specified the presence of 22 carbon resonances counting those of one carbonyl carbon at δ_C 174.5, one methine olefinic carbon at δ_C 130.0, one non-

protonated olefinic carbon at δ_C 141.5, two oxygenated carbons at δ_C 70.5 and 70.3, one methine carbon at 36.3, fourteen methylene carbons at δ_C 65.2, 71.9, 72.6, 29.6, 64.4, 71.8, 33.5, 25.0, 34.2, 27.3, 29.8, 22.7, and 32.0, along with two methyl carbons at δ_C 14.1. The typical ^{13}C NMR signal recorded at δ_C 174.5 was assigned to the ester carbonyl carbon. The significantly deshielded methylene protons at δ_H 4.20 (H-15) displayed strong HMBC cross peaks with the carbonyl carbon at δ_C 174.5, which supported that it should be adjacent to the electronegative oxygen atom of the ester functionality. The long range correlations between δ_H 3.61 (H-13) and δ_C 72.6 (C-11), and that between δ_H 3.66 (H-6) and δ_C 64.4 (C-8) supported the location of the ether bridges in the structure. The methine signal at δ_C 36.3 was in agreement with the deshielded proton signal at δ_H 2.17, which referred to a possible olefinic group in the vicinity. This attribution was appropriately validated by the HMBC cross peak between the carbon signal and the olefinic proton at δ_H 5.34. The carbonyl group at the C-1 position of the studied compound caused intense deshielding of the methylene proton at δ_H 2.35, which appeared as a triplet, and therefore, has been consigned the C-2 position of the structure. Detailed HMBC couplings established the closed loop of the 10, 14-dihydroxy-7, 12-dioxapentadecanolide moiety in the compound. The one bond ^1H-^1H COSY recorded three spin systems within the dioxapentadecanolide moiety. The attachment of an alkenyl side chain to the macrocyclic lactone moiety was predicted from the long range HMBC cross peaks.Further HMBC couplings found within the alkenyl chain were δ_H 5.34 to δ_C 27.3 (C-3′); δ_H 2.02 to δ_C 29.7 (C-4′); δ_H 1.31 to δ_C 22.7 (C-5′); δ_H 1.26 to δ_C 29.7 (C-4′), 14.1 (C-6′); δ_H 0.88 to δ_C 22.7 (C-5′); δ_H 1.30 to δ_C 14.1 (C-1′c); and δ_H 0.88 to δ_C 32.0 (C-1′a). The three spin systems δ_H 5.34 (H-2′)/2.02 (H-3′); δ_H 1.26 (H-5′)/0.88 (H-6′), and that at δ_H 2.02 (H-1′a)/1.30 (H-1′b)/0.88 (H-1′c) additionally attributed the side chain structure.

Compound BS-2

10,14-dihydroxy-4-(1'propyl-1'(Z)-hexen-1'-yl)-7,12-dioxapentadecanolide



Sample yield	0.091 % based on dry weight
Physical description	Yellowish oil
Molecular formula	$C_{22}H_{40}O_6$
Molecular weight	400.2825

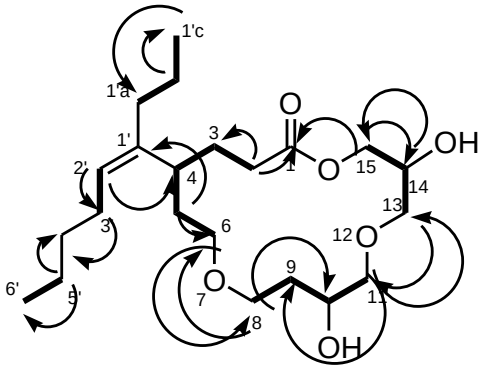


Fig. 5.2.1.2. Key ^{1}H-^{1}H COSY and HMBC correlations of ***10, 14-dihydroxy-4-(1'propyl-1'(Z)-hexen-1'-yl)-7,12-dioxapentadecanolide*** (**BS-2**). The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; the HMBC couplings are indicated as double barbed arrow.

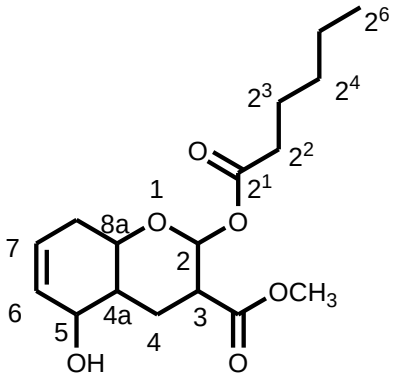
5.2.1.3. Structural Characterization of Compound BS- 3

The purified compound SB-3 was obtained as a yellow oily liquid. Molecular formula of $C_{17}H_{26}O_6$ was established by mass spectrometry [m/z 326.1732] for SB-3, and supported by 1H and 13C NMR data. Infrared absorptions of 3400 and 1758 cm-1 indicated the presence of hydroxyl and ester carbonyl functionalities. The molecular formula suggested 5 degrees of double bond equivalence, which were due to an alkene and two ester carbonyls (in conjunction with two rings) based on the characteristic chemical shifts of protons and carbons (^{1}H and ^{13}C NMR) and previously reported NMR values. Aromatic region (δ 6.5–8.5) of the compound did not exhibit any notable signals, which confirmed the absence of aromatic moiety. The ^{13}C NMR exhibited two peaks at δ 173.29 and 174.29 which were confirmed as quaternary carbon based on the distortionless enhancement by polarization transfer (DEPT) analysis. It was evident from the fact that the chemical shift in the range δ 170–185 apparently forms a part of the carbonyl group, preferably ester moiety, and thus the carbon at δ 173.29 and 174.29 was attributed to ester carbon. The methine carbon at δ 98.2 (C-2) exhibited significant downfield shifts, and attributed to the carbon adjacent to an electronegative oxygen atom. Further inspection of the 13C NMR and DEPT spectral data indicated that SB-3 contained two methyl groups at δ 14.1and 52.7 ppm, six methylene

groups at δ 24.89, 31.94, 34.08, 29.7, 29.37 and 22.7ppm. Methine carbon peaks were found at δ 98.2, 49.13, 43.76, 68.95, 130.0, 120.66 and 71.03ppm. Thus interpretation of ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC NMR data allowed assignment of SB-3 as 2-(butyryloxy)-5-hydroxy-3,4,4a,5,8,8a-hexahydro-2H-chromene-3- methyl carboxylate.

Compound BS-3

2-(butyryloxy)-5-hydroxy-3,4,4a,5,8,8a-hexahydro-2H-chromene-3- methyl carboxylate



2-(butyryloxy)-5-hydroxy-3,4,4a,5,8,8a-hexahydro-2H-chromene-3- methyl carboxylate	
Sample yield	0.178 % based on dry weight
Physical description	White crystalline solid
Molecular formula	$C_{17}H_{26}O_6$
Molecular weight	326.1732

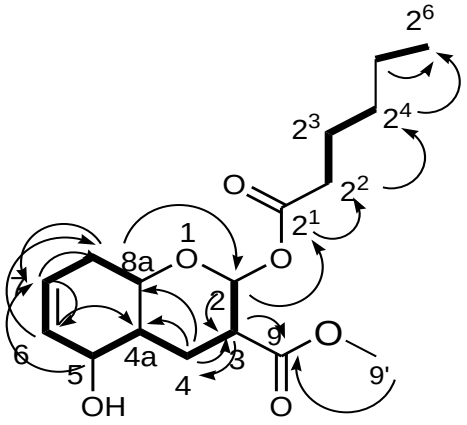


Fig. 5.2.1.3. Key ^{1}H-^{1}H COSY and HMBC correlations of *2-(butyryloxy)-5-hydroxy-3,4,4a,5,8,8a-hexahydro-2H-chromene-3- methyl carboxylate* (**SB-3**). The

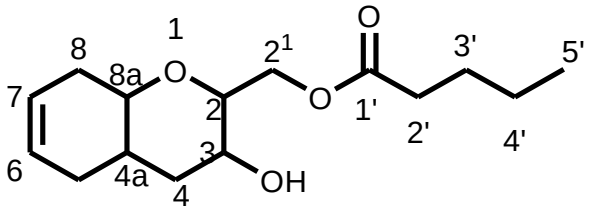
key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; the HMBC couplings are indicated as double barbed arrow.

5.2.1.4. Structural Characterization of Compound BS-4

The compound **BS-4**, ***(3-hydroxy-3,4,4a,5,8,8a-hexahydro-2H-chromen-2-yl)methyl pentanoate*** was isolated as yellow oil. Its molecular ion peak appeared at *m/z* 268, which along with extensive NMR studies confirmed the molecular formula of the title compound as $C_{15}H_{24}O_4$. This exhibited four double bond equivalences with two ring systems, an alkenic double bond and one ester carbonyls based on the characteristic chemical shifts of protons and carbons (^{1}H and ^{13}C NMR) supported by previously reported NMR values. IR absorptions were observed at around 3400 cm^{-1}, suggesting the presence of hydroxy groups. The structure of this compound was deduced from its ^{13}C NMR and DEPT spectroscopic data which showed that the compound has 15 carbons, including one methyl, seven sp^3 methylenes, four sp^3 methines (including three oxymethine), two sp^2 methines, and one sp^2 quaternary carbons. From the ^{1}H NMR spectrum of BS-4, resonances of two olefinic methine proton (δ 5.38 and 5.35 Hz), were observed. The HSQC, HMBC along with ^{1}H-^{1}H COSY correlation analyses confirmed the structure.

Compound BS-4

(3-hydroxy-3,4,4a,5,8,8a-hexahydro-2H-chromen-2-yl)methyl pentanoate



Sample yield	1.357 % based on dry weight
Physical description	Yellow oily
Molecular formula	$C_{15}H_{24}O_4$
Molecular weight	268.1679

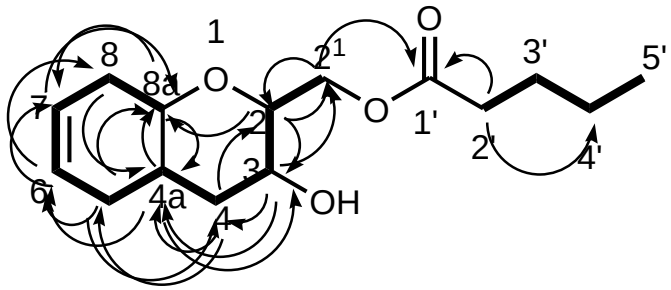


Fig. 5.2.1.4. Key ^{1}H-^{1}H COSY and HMBC correlations *(3-hydroxy-3,4,4a,5,8,8a-hexahydro-2H-chromen-2-yl)methyl pentanoate* (**BS-4**). The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; the HMBC couplings are indicated as double barbed arrow.

5.3. Antioxidant and Anti-inflammatory Activities of Secondary Metabolites Isolated from *B. spirata*

The *in vitro* antioxidant properties of the four studied compounds were evaluated in accordance with their capacity to scavenge DPPH and ABTS+ radicals, and the target bioactivities were compared with the commercial antioxidant α-tocopherol. Among the two studied secondary metabolites from *B. spirata*, the compound BS-2 characterised as 10,14-dihydroxy-4-(1′propyl-1′-hexen-1′-yl)-7,12-dioxapentadecanolide proved to be a better antioxidant showing potential scavenging activity against the free radicals in vitro (IC_{50} DPPH 0.23 mg/mL; IC_{50} ABTS 0.26 mg/mL), and the antioxidant activities were found to be higher than α-tocopherol (IC_{50} DPPH 0.60mg/mL; IC_{50} ABTS 0.73 mg/mL). The antioxidant activities of the studied compounds as determined against DPPH and $ABTS^{+}$ were in the order: **BS-2> BS-1> BS-3>BS-4.** The hypoglycemic effects of the isolated compounds were determined *in vitro* through the inhibition of carbohydrate hydrolyzing enzymes α-amylase and α-glucosidase. Inhibition of these enzymes would retard the absorbance of carbohydrates in the intestine, resulting in the control of postprandial hyperglycemia (Bischoff, 1994). The 2*H*-chromenyl derivative 2-(butyryloxy)-5-hydroxy-3, 4, 4a, 5, 8, 8a-hexahydro-2*H*-chromene-3-methyl carboxylate (BS-3) described in this study showed greater α-amylase and α-glucosidase inhibitory activities (IC_{50} < 0.5mg/mL) than the rest of the compounds The positive control acarbose exhibited the IC_{50} values of 0.28 and 0.31 mg/mL for α-amylase and α-glucosidase inhibitory activities, respectively. The anti-diabetic property of the isolated compounds against α-glucoxidase enzyme were in the order of **BS-3> BS-1> BS-2> BS-4**. Selective inhibition of 5-lipoxygenase (5-LOX) is a preferential method to deter the inflammatory stimuli. The anti-inflammatory activities of the purified compounds as inferred

by the 5-lipoxygenase assay showed that the polyether macrocyclic lactone **BS-2** had greatest anti-inflammatory potentials (IC_{50} 0.20 mg/mL).The anti-inflammatory potential of isolated compounds determined against 5-LOX enzyme were in the order as **BS-2 > BS-3>BS-4> BS-1**. There has been earlier report that the column purified fractions of the methanol extract of the Babylonidae *Babylonia zeylanica* could significantly reduce the carrageenan-induced paw edema in albino rats when compared to the control diclofenac sodium (Santhi et al., 2012). Likewise, the gastropod molluscs *Trochus tentorium* and *Drupa margariticola* were found to be potent inhibitors of exudative and proliferate phase of inflammation (Chellaram and Edward, 2009 a,b).

CHAPTER 6: ISOLATION AND CHARACTERIZATION OF BIOACTIVE SECONDARY METABOLITES FROM *CHICOREUS RAMOSUS*

6.1. Sample Preparation

The samples of the marine gastropod *C. ramosus* were collected from the fishing harbours of Tuticorin situated along the south-east coast of Tamil Nadu in the Gulf of Mannar region, located between India and Sri Lanka (latitude 8°48'N and longitude 78°09'E). The shells were slivered to detach the soft body tissue, which were cleansed with sterile distilled water to remove the externalities. A total of 4.75 kg of *C. ramosus* meat were obtained, and those were cut into small pieces, macerated and freeze dried by utilizing a laboratory freeze-drier (Martin Christ alpha 1-4 LDplus, Martin Christ, Germany) yielding lyophilized powder of 1070 g. The lyophilized powder (500 g each) of each gastropod was sonicated with solvent chloroform (500 mL X 2) and EA-MeOH (1:1 v/v, 2 x 500 mL), for 2 h under an inert atmosphere of N_2 before being sifted through Whatman filter paper No. 1 to acquire the clarified filtrates (1 L). The clarified filtrates were dried through Na_2SO_4 (100 g), and evaporated (45 ^oC) by means of a rotary evaporator (Heidolph Instruments GmbH and Co., Schwabach, Germany) under vacuum to get orange-yellow viscous residues. The yields of the EtOAc-MeOH and chloroform extract based on dry material were 11.54 and 1.70 percent, respectively for *C. ramosus*.

6.1.2. Chromatographic purification and spectral analysis of pure compounds from *C. ramosus*

The EtOAc-MeOH extract (70 g) of the muricd *C. ramosus* was subjected to column chromatography over a silica gel column (60–120 mesh; 5 × 150 cm) with stepwise gradient of *n*-hexane, ethyl acetate and methanol as eluting solvents to afford ten major fractions (CR_A-CR_J) based on TLC analysis. The major column fractions (CR_A-CR_J) were assayed for antioxidant, antidiabetic and anti-inflammatory activities. The fractions CR_B, CR_E, CR_F and

CR_H were selected for further purifications on the basis of their greater antioxidant {2,2-diphenyl-1-picrylhydrazyl (DPPH)/2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^+$) scavenging} properties. The fraction CR_B, which was eluted at 20% *n*-hexane-EtOAc (1.6 g), was column chromatographed on silica gel (60-120 mesh) eluting with *n*-hexane-EtOAc (90:10 to 0:100, v/v) gradient to give six major fractions CR_B1-6 after thin layer chromatography (TLC). The fraction CR_B3 (413.3 mg), which was obtained by eluting with *n*-hexane: EtOAc (3:1, v/v), being a mixture was flash chromatographed (Biotage, 230–400 mesh, 12 g; Sweden, Biotage No. 25+M 0489-1) at a collection UV wavelength of 254 nm using a stepwise gradient of *n*-hexane/EtOAc (0-5% EtOAc) to afford 18 fractions (15 mL each). The fractions with similar TLC patterns were combined together to give seven pooled fractions (CR_B3-1–CR_B3-7). The fraction CR_B3-2 (220 mg) on subsequent flash chromatographic purification with the eluent polarity initiating from 100% *n*-hexane followed by EtOAc and methanol afforded compound CR-**1** (70 mg). The fraction CR_E (70 mg) was eluted with EtOAc/MeOH (1:1, v/v) on silica gel to afford 15 fractions, which were summarized to eight fractions, following TLC analysis. The sub-fraction CR_E6 being a mixture showing greater activity than the other subfractions, was further purified by preparative HPLC using methanol/acetonitrile (MeOH/MeCN 90:10, v/v) as the mobile phase (at a flow rate of 6.0 mL/min), to afford compound **CR-2** (40 mg). The fraction, CR_F on chromatographic purification yielded twelve sub-fractions, CR_{F-1} to CR_{F-12}. Among these, the sub-fraction CR_F4 and CR_F 10 were found to possess potential antioxidant and anti-inflammatory activities ($IC_{50} \leq 1$ mg/mL) when compared to other fractions and hence shortlisted for further downstream purifications (Table S1). The sub-fraction CR_F4 was additionally subjected to flash chromatographic fractionation, which led to the isolation of the pure compound **CR-3** (0.36 g). The fraction CR_F4 on preparative TLC purification using 15% MeOH/$CHCl_3$ resulted compound **CR-4** (49 mg). The main fraction CR_H displaying higher antioxidant activities ($IC_{50} < 1$ mg/mL) was fractionated using EtOAc-MeOH (90:10 to 0:100, v/v) to give seven sub-fractions, CR_H1 to CR_H7. Out of these the fractions CR_H4 and CR_H7 were shortlisted for further downstream purification due to higher bioactivities and greater yield. The fraction CR_H4 was subjected to bioassay guided repetitive column chromatographic purifications using silica of finer mesh size (230-400 mesh) eluting with solvents of increasing polarity to obtain the pure compound CR-5 (54.6 mg) which was eluted using 5% EtOAc /MeOH through a 230-400 meshed silica. Reverse phase purification of the sub-fraction CR_H7 (180 mg) which was eluted at 80% EtOAc /MeOH on further RP-

C_{18}-HPLC using MeOH/ACN (8:2 v/v, R_t 2.50) yielded studied pure compound **CR-6** (66 mg) as a major component.

6.1.3. Bioactive potentials of secondary metabolites isolated from *C. ramosus* and structure-activity correlation analyses

The bioactive potentials of six pure compounds isolated from *C. ramosus* were checked by various *in vitro* antioxidants (DPPH and $ABTS^+$) and anti-inflammatory (5-LOX) assays and their bioactivities were expressed as IC_{50} values in mg/mL concentrations.

6.1.4. Structure activity relationship studies by molecular descriptors

Different physicochemical parameters of the purified compounds were analyzed to study the structure–activity relationship. The structural descriptors, such as steric (molar volume, molar refractivity), hydrophobic (log P_{ow} logarithmic value of the octanol–water partition coefficient) and polarizability (electronic descriptor) factors were acquired from the ChemDraw Ultra Natural Product Research 11 (8.0 database).

6.1.5. Statistical analysis

One way analysis of variance (ANOVA) was carried out with the Statistical Program for Social Sciences 13.0 (SPSS, ver. 13.0, SPSS Inc., Quarry Bay, HK) to assess for any significant differences between the means. The significant differences were represented as $P<0.05$ and the values were given as mean of triplicate standard deviation. Statistical analyses were based upon different bioactivities of the pure compounds *vis-à-vis* standards, with regard to the antioxidant, antidiabetic and anti-inflammatory activities.

6.2. Results and Discussion

6.2.1. Chromatographic purification and spectral analysis of secondary metabolites from *C. ramosus*

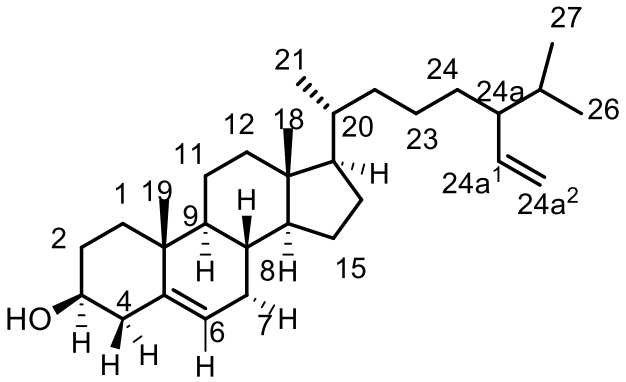
Repeated chromatographic purifications of the solvent fractions obtained from *C. ramosus* led to the isolation of six previously disclosed secondary metabolites. The novelty of isolated compounds has been verified with those described in the SciFinder databases. The structures were elucidated and confirmed by extensive analysis of the the ^{1}H NMR and ^{13}C NMR, including two-dimensional nuclear magnetic resonance spectroscopy (2D NMR) experiments such as heteronuclear single-quantum correlation spectroscopy, HSQC; correlation spectroscopy (^{1}H-^{1}H COSY); heteronuclear multiple-bond correlation spectroscopy (HMBC) and nuclear Overhauser effect spectroscopy (NOESY) along with mass spectra and Fourier transform infrared (FTIR) analyses.

6.2.1.1. Structural Characterization of Compound CR-1

It was isolated as a white amorphous solid (m.p.140-142 ^{0}C) upon repeated chromatography over silica gel. The molecular formula was determined to be $C_{30}H_{50}O$ from the EIMS (*m/z* 426) and HR-ESI-MS data (*m/z* 427.3869 $[M+H]^+$, calcd for $C_{30}H_{51}O$, 427.3862) which indicated six degrees of unsaturation. With two olefinic residues deduced from the ^{13}C NMR experiment, the remaining unsaturations were thus accounted for the ring systems. With the help of extensive NMR experiments, the structure of the compound was deduced as a Δ^5 cholestenol analogue (5*Z*)-24a-homo-cholesta-5, 24a^1 (24a^2)-dien-3-β-ol (**1**) with an ethylene group branching from the homo carbon atom (C-24a).). A multiplet found at the downfield position of δ 3.50 (m, 1H) appeared to correspond to the proton germinal to the hydroxyl group found at C-3 in 3β hydroxyl Δ^5-sterols. The compound had two singlet methyl signals corresponding to other reported sterols from molluscs which had two singlet methyls at C-18 and C-19 positions. The highly deshielded proton resonances at δ 5.35 which showed attachment to the methine carbon in the HSQC spectra at δ121.9 were classical of the endocyclic olefin protons placed at C-6 in the Δ^5 sterols. The high field positions of the unusual proton signals at δ 5.8 (m,1H), δ 4.97 (dd, *J*= 3.86, 12.0, Hz) and δ4.91(dd, *J*= 3.86, 16, Hz) were indicative of an extra olefinic group in the structure. The hexacyclic and pentacyclic frameworks of the steroid nucleus were supported by the HMBC correlations in conjugation with the ^{1}H-^{1}H COSY spectrum.

Compound CR-1

5(Z)-24a-Homo-cholesta-5, 24a^1 (24a^2)-dien-3-β-ol



Sample yield	0. 09 % based on dry weight
Physical description	white amorphous solid
Molecular formula	$C_{30}H_{50}O$
Molecular weight	426.3862

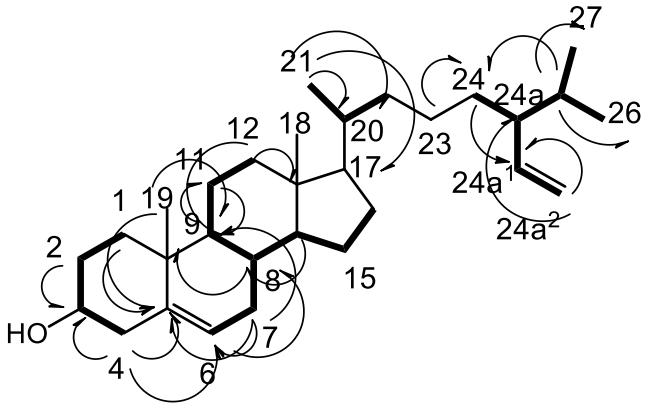


Fig. 6.2.1.1. Key ^{1}H-^{1}H COSY and HMBC correlations *of 5(Z)-24a-Homo-cholesta-5, 24a^{1} (24a^{2})-dien-3-β-ol (1).* The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; the HMBC couplings are indicated as single barbed arrow.

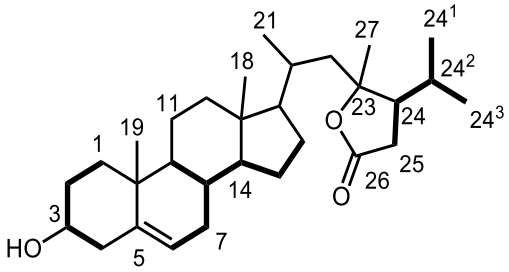
6.2.1.2. Structural Characterization of Compound CR-2

A steroidal lactone analogue derived as white crystalline solid, was characterized as a 27(25 → 23)-abeo-(5*Z*)-3β-hydroxy-24-isopropyl cholesteno-26, 23-lactone (**2**). The molecular ion peak at *m/z* 456 in conjunction with the extensive NMR spectroscopic evidence proposed its molecular formula as $C_{30}H_{48}O_3$, which implied seven degrees of unsaturation. The compound was found to enclose one olefinic residue and one carbonyl moiety deduced from the ^{13}C NMR experiment. The ^{13}C NMR spectrum deduced an overall 30 signals, including the peaks at δ 72.0; 81.9 and 177.5, which were presumed to be directly attached to oxygen due to their downfield position. The ^{13}C NMR spectrum in connection distortionless enhancement by polarisation transfer ($DEPT_{135}$) experiment exhibited the resonances due to six methyls, ten methylenes, nine methine resonances, whereas the remaining five signals were attributed to non-protonated carbon atoms. Six methyl signals were found at δ 12.0, 19.4, 18.8, 21.2, 23.0 and 22.8, whereas the carbon peaks due to olefinic methine were found at δ 121.9, and the quaternary carbon associated with this exhibited its signal at δ 140.9 in the ^{13}C NMR spectrum. In addition to the typical quaternary carbon signals of a Δ^5 sterol which were found at δ 36.6 (C-10), 42.4 (C-13) and δ 140.9 (C-5), a high field signal pertaining to a quaternary carbon was apparent at δ 81.8. The ^{1}H NMR spectrum of the compound displayed two characteristic singlet methyl signals at δ 0.68 and δ 1.01 corresponding to the angular methyl groups C-18 and C-19, respectively. However this compound had an additional singlet at δ 1.46 attributed to a methyl carbon at δ 21.2 unlike usual sterols. There

were three spin systems within the molecule as exhibited by the ^{1}H-^{1}H COSY correlations. The steroidal core rings ABCD enclosed the first two spin systems. Furthermore, the long range HMBC correlations established the presence of the cyclopentenophenanthrene ring system. The HMBC correlations and the nmr data illustrated the presence of four steroidal rings, a double bond between C5 and C-6 and a carbonyl carbon at δ177.5 corresponding to an ester group. These facts together with the degree of unsaturation as per the mass spectral value proposed the incidence of an additional ring within the side chain of the molecule. The assignment of the proton and carbon signals within the side chain was established using the HMBC correlations which led us to propose the lactonisation of the ester carbonyl in the side chain. The high field quaternary carbon at δ 81.8 which was presumed to be the O-linked carbon atom of the lactone ring, exhibited strong HMBC correlations with the methylene (δ 2.00) and methine protons (δ1.52) at C-22 and C-20 positions respectively. Accordingly the oxygenated quaternary carbon was placed at C-23 position. The presence of the carbolactone ring and an extra methyl group in the side chain made the sterol molecule more analogous to a modified cholestenoic acid derivative alkylated at C-24 with an isopropyl group. As the methyl group found in the cholestenoic acid is originally at C-25, which has now shifted to C-23 in the present structure, we have proposed the nomenclature of this new cholestenolactone as 27(25 → 23)-abeo-(5*Z*)-3β-hydroxy-24-isopropyl cholesteno-26, 23-lactone which we called under the trivial Ramosolide.

Compound CR-2

27(25→23)-abeo-(5Z)-3β-hydroxy-24-isopropyl cholesteno-26, 23-lactone



Sample yield	0.05 % based on dry weight
Physical description	White crystalline
Molecular formula	$C_{30}H_{48}O_3$
Molecular weight	456.3603

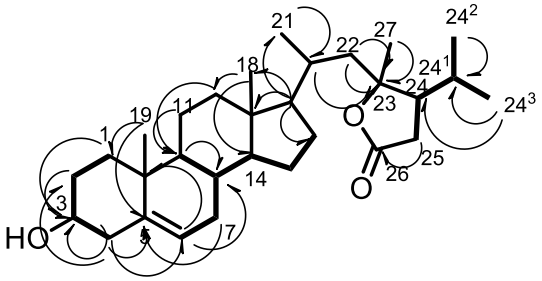


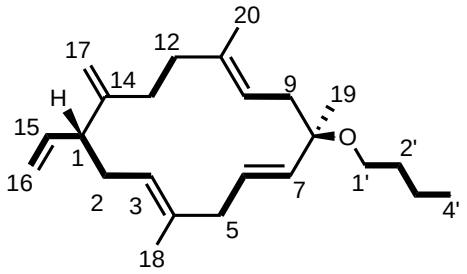
Fig. 6.2.1.2. Key ^{1}H-^{1}H COSY and HMBC correlations of *(5Z)-3β-hydroxy-5-en-23-methyl cholestryl-23, 24^2-lactone***.** The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; the HMBC couplings are indicated as single barbed arrow.

6.2.1.3. Structural Characterization of Compound CR-3

The compound CR-3 was isolated as a yellow oily liquid on repetitive column chromatography. The elemental composition was found to be $C_{24}H_{38}O$, which suggested six degrees of unsaturation including one ring system and five double bonds. Its molecular mass was recorded at *m/z* 342 by mass experiments. The ^{13}C spectrum along with DEPT displayed the resonances for 24 carbon atoms, which comprised of four ten methylenes, quaternary carbons, four methyls and six methines. The characteristic groups corresponding to one oxygenated methylene (δ_H 3.40/δ_C 64.60); two trisubstituted alkenes {(δ_C 135.87; δ_H 5.20/δ_C 125.49); (δ_C 139.07; δ_H 5.12/δ_C 120.64)}; one disubstituted alkene (δ_H 5.74/δ_C 124.76; δ_H 5.60/δ_C 129.83); one disubstituted end methylene (δ_C 151.93; δ_H 4.70, 4.67/δ_C 115.06) and one monosubstituted terminal vinylic group {(δ_H 5.76/δ_C 139.29); (δ_H 4.96, 4.94/δ_C 114.06)} were identified by ^{1}H, ^{13}C and HSQC values. The ^{1}H-^{1}H correlation spectroscopy identified five spin systems, which suggested different partial structures, A, B, C, D and E. The fragments, A and B were allied from side to side via an sp^2 hybridized carbon δ 135.87 (C-4), as reviewed by HMBC connections. The B and C fragments were linked through an sp^3 hybridized quaternary carbon at δ 83.97 (C-8) as judged by the heteronuclear linkages. The spin systems, C and D were related via the sp^2 hybridized carbon at δ 139.07 (C-11) as per the HMBC correlations which led to the closure of fragments and the formation of 14-membered macrocyclic system. HMBC correlations additionally supported the presence of exocyclic double bonds, which were appropriately positioned at C-14. The sp^3 hybridized quaternary carbon at δ 83.97 (C-8) was deduced to be cross-linked to the oxygenated methylene at δ 3.40 (H-1′). This oxygenated methylene was found to be the part of spin system E (butoxy chain), and that suggested the attachment of butoxy moiety at C-8 of basic 14-membered macrocyclic skeleton.

Compound CR-3

(3E, 6E, 10E)-8a-butoxy-17(15→14), 20(12→11)-bis-abeo-cembra-3,6,10,14(17),15-pentaene



Sample yield	0.09 % based on dry weight
Physical description	yellow oily liquid
Molecular formula	$C_{24}H_{38}O$
Molecular weight	342.2934

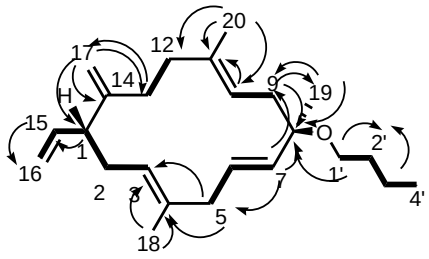


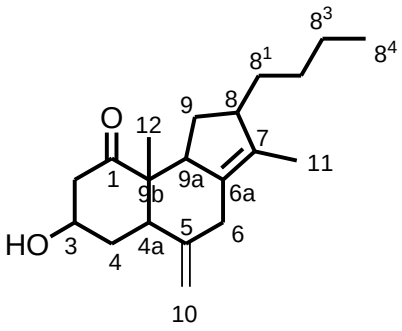
Fig. 6.2.1.3. Key ^{1}H-^{1}H COSY and HMBC correlations of *(3E, 6E, 10E)-8a-butoxy-17(15→14), 20(12→11)-bis-abeo-cembra-3,6,10,14(17),15-pentaene* (CR-**3**). The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; the HMBC couplings are indicated as double barbed arrow.

6.2.1.4. Structural Characterization of Compound CR-4

This new meroterpenoid was isolated as amorphous white colored solid upon repeated chromatography. Its mass spectrum exhibited a molecular ion peak at *m/z* 316.2406 (HRESIMS *m/z* 317.2494 amu $[M+1]^{+}$), which in combination with its ^{1}H and ^{13}C NMR data indicated the elemental composition as 7-hydroxy-3,9a-dimethyl-5-methylene-2-pentyl-4,5,5a,6,7,8,9a,9b-octahydro-1H-cyclopenta[a]naphthalen-9(2H)-one ($C_{21}H_{32}O_2$) with six degrees of unsaturation. Three degree of unsaturation from the double bond equivalency and the other remaining degree of unsaturation was owing to ring systems.

Compound CR-4

7-hydroxy-3,9a-dimethyl-5-methylene-2-pentyl-4,5,5a,6,7,8,9a,9b-octahydro-1H-cyclopenta[a]naphthalen-9(2H)-one



Sample yield	0.07 % based on dry weight
Physical description	Amorphous white solid
Molecular formula	$C_{21}H_{32}O_2$
Molecular weight	316.2404

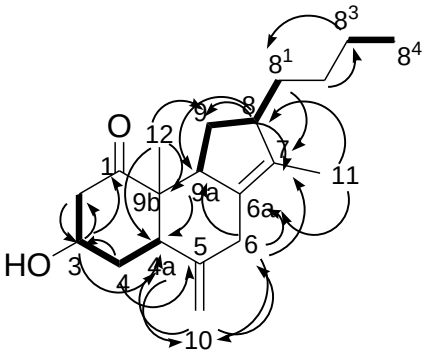


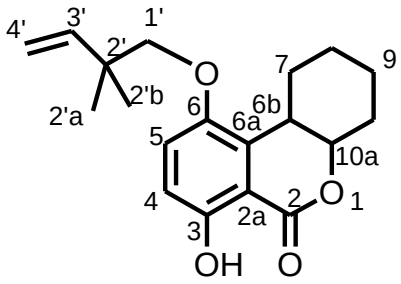
Fig. 6.2.1.4. Key ^{1}H-^{1}H COSY and HMBC correlations *of 7-hydroxy-3,9a-dimethyl-5-methylene-2-pentyl-4,5,5a,6,7,8,9a,9b-octahydro-1H cyclopenta[a]naphthalen-9(2H)-one* **(CR-4).** The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; the HMBC couplings are indicated as double barbed arrow.

6.2.1.5. Structural Characterization of Compound CR-5

This new meroterpenoid was isolated as yellow color oily liquid upon repeated chromatography. Its mass spectrum exhibited a molecular ion peak at *m/z* 316.1679 (HRESIMS *m/z* 317.1769 amu $[M+H]^+$), which in combination with its ^1H and ^{13}C NMR data indicated the elemental composition 6-(2',2'-dimethyl)-3'-en-1'-yl-1'-oxy)-3-hydroxy-1H-benzo[c]chromene-2*(10aH)*-one ($C_{19}H_{24}O_4$) with eight degrees of unsaturation. Five degree of unsaturation from the double bond equivalency and the other three degree of unsaturation was due to a cyclic ring system. The total numbers of carbon atoms were found to be 19 from the detailed ^{13}C and DEPT analyses.

Compound CR-5

6-(2',2'-dimethyl)-3'-en-1'-yl-1'-oxy)-3-hydroxy-1H-benzo[c]chromene-2(10aH)-one



Sample yield	0.078 % based on dry weight
Physical description	Yellow oily liquid
Molecular formula	$C_{19}H_{24}O_4$
Molecular weight	316.1679

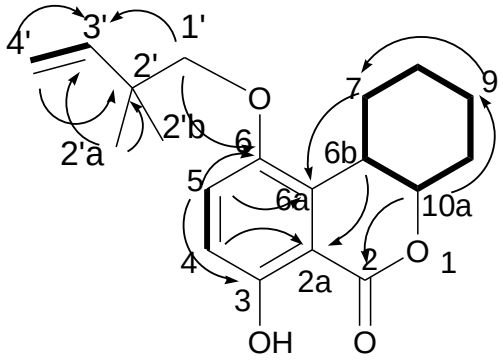


Fig. 6.2.1.5. Key ^1H-^1H COSY and HMBC correlations of ***6-(2', 2'-dimethyl)-3'-en-1'-yl-1'-oxy)-3-hydroxy-1H-benzo[c]chromene-2(10aH)-one*** (C**R-5**). The

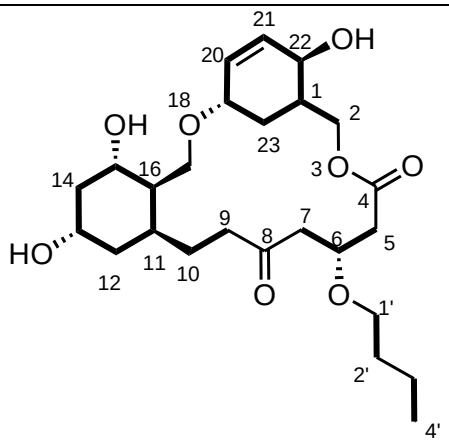
key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; the HMBC couplings are indicated as double barbed arrow.

6.2.1.6. Structural Characterization of Compound CR-6

Compound **CR-6**, a fused macrocyclic lactone was isolated as a brown liquid. Its molecular formula, $C_{25}H_{38}O_8$, was deduced from the MS (*m/z* 466 $[M]^+$) and ^{13}C NMR spectroscopic data, showing seven indices of hydrogen deficiency. Two degree of unsaturation were from two double bonds and two were identified as due to two carbonyl bonds, and the remaining degrees of unsaturation were accounted as due to cyclic ring systems. The IR spectrum revealed the presence of a hydroxyl (3403cm^{-1}), ester carbonyl (1749 cm^{-1}) and olefinic (1558cm^{-1}) groups. The strong infrared absorption bands between 1250 and 1050 cm^{-1} (C–O-C stretch) were indicative of the presence of the ether bridges in the studied compound. The ^{13}C NMR chemical shifts alongside DEPT spectral data displayed the presence of ten methylene carbons, twelve methine, one methyl and two quaternary carbon atoms. The assignments were based on the key ^{1}H-^{1}H COSY and HMBC correlations.

Compound CR-6

6-(butoxy)-13,15,22-trihydroxy-3,18-dioxatricyclo[18.3.1.0^{11,16}]tricosa-20-ene-4,8-dione



Sample yield	0.095 % based on dry weight
Physical description	brown liquid
Molecular formula	**$C_{25}H_{38}O_8$**
Molecular weight	466.2570

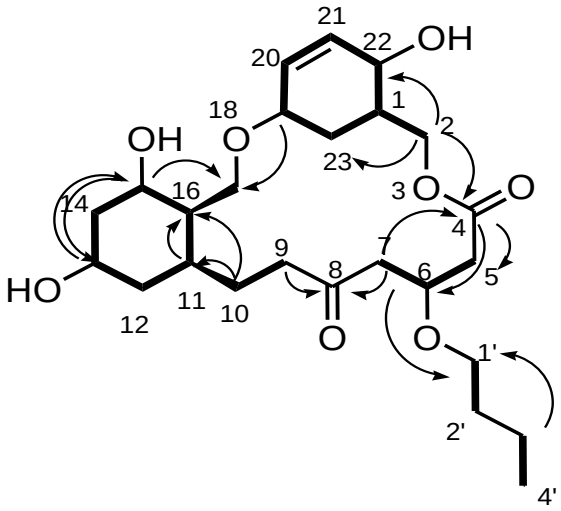


Fig. 6.2.1.4. Key ^{1}H-^{1}H COSY and HMBC correlations of *6-(butoxy)-13,15,22-trihydroxy-3,18-dioxatricyclo[18.3.1.011,16]tricosa-20-ene-4,8-dione* **(CR-6)**. The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; the HMBC couplings are indicated as single barbed arrow.

6.3. Bioactivities of Secondary Metabolites Isolated from *C. ramosus* and SAR analyses

This section describes the antioxidant, antidiabetic and anti-inflammatory potentials of the secondary metabolites isolated from *Chicoreus ramosus* and their activities expressed in their IC_{50} values. Bioassay guided chromatographic fractionation of solvent fractions of *C. ramosus* afforded potential secondary metabolites. The pharmacological properties comprising of antioxidant, anti-inflammatory and antidiabetic potentials of the purified compounds from the organic extract of *C. ramosus* were shown as graphical representation, and their activities were judged against the commercialized antioxidative agents and non-steroidal anti-inflammatory drugs (NSAIDs). The *in vitro* antioxidant properties of the six studied compounds were evaluated in accordance with their capacity to scavenge DPPH and $ABTS^{\cdot+}$ radicals, and the target bioactivities were compared with the commercial antioxidant α-tocopherol. Among the studied secondary metabolites from *C. ramosus*, the tricyclic polyketide, characterized as 6-(butoxy)-13,15,22-trihydroxy-3,18-dioxatricyclo[18.3.1.$0^{11,16}$]tricosa-9,20-diene-4,8-dione was demonstrated to be a better antioxidant showing potential scavenging activity against the free radicals in vitro (IC_{50}

DPPH 0.24 mg/mL; IC_{50} ABTS.+ 0.26 mg/mL), and the antioxidant activities were found to be higher than α-tocopherol (IC_{50} DPPH 0.60mg/mL; IC_{50} ABTS 0.73 mg/mL). The antioxidant activities of the studied compounds as determined against DPPH and ABTS.+ were in the order: CR-6> CR-3> CR-5> CR-4> CR-2 > CR-1. The hypoglycemic effects of the isolated secondary metabolites were determined *in vitro* through the inhibition of carbohydrate hydrolyzing enzymes *α*-amylase and *α*-glucosidase. Inhibition of these enzymes would retard the absorbance of carbohydrates in the intestine, resulting in the control of postprandial hyperglycemia (Bischoff, 1994). The 1*H*-benzochromenone characterized as 6-(2′,2′-dimethyl)-3′-en-1′-yl-1′-oxy)-3-hydroxy-1*H*-benzo[c]chromene-2(10a*H*)-one showed greater α-amylase and α-glucosidase inhibitory activities (IC_{50} < 0.6 mg/mL) than the remaining compounds. The positive control acarbose exhibited the IC_{50} values of 0.28 and 0.31 mg/mL for α-amylase and α-glucosidase inhibitory activities, respectively. The anti-diabetic property of the isolated compounds against α-glucoxidase enzyme were in the order of CR-5> CR-4> CR-6 > CR-3> CR-1 > CR-2. Selective inhibition of 5-lipoxygenase (5-LOX) enxyme is a preferential method to prevent the inflammatory stimuli. The anti-inflammatory activities of the purified compounds as inferred by the 5-lipoxygenase assay showed that the macrocyclic polyketide displayed significantly greater attenuation property against pro-inflammatory 5-lipoxidase (5-LOX, IC_{50} 0.44 mg/mL) than that displayed by the non-steroidal anti-inflammatory drugs ibuprofen (IC_{50} 0.93 mg/mL) (P < 0.05). The anti-inflammatory potential of isolated compounds determined against 5-LOX enzyme were in the order as: CR-6> CR-5> CR-2> CR-4> CR-3 > CR-1. There has been earlier studies of the promising anti-inflammatory activities exhibited by the muricid gastropods (Santhi et al., 2011).

CHAPTER 7: ISOLATION AND CHARACTERIZATION OF BIOACTIVE POLYSACCHARIDE FROM *BABYLONIA SPIRATA*

7.1. Materials and Methods

This section described the instruments used for the present study along with the reagents and solvents used.

7.1.1. Sample Preparation

The crude polysaccharide has been extracted from the marine gastropods using water extraction method. The crude polysaccharides isolated from *C. ramosus* and *B. spirata* were checked for the bioactive potentials by various *in vitro* antioxidants (DPPH and $ABTS^{+}$) and

anti-inflammatory (5-LOX) assays and their bioactivities were expressed as IC_{50} values in mg/mL concentrations. On the basis of the bioasssays, the crude polysaccharide from *B. spirata*, which showed higher bioactive potentials was further fractionated by ion exchange chromatography on a DEAE-52 column.

7.1.2. Chromatographic Purification and Spectral Analysis of polysaccharide from *Babylonia spirata*

The crude CPSBS was sequentially purified by chromatography of a DEAE-52 column and according to the reported methods with slight modifications. The 50 mg crude CPSBS was dissolved in 1.5 mL deionized water and loaded onto a DEAE-52 column (2.6 × 70 cm). Then, the column was sequentially eluted with distilled water and gradient eluted with NaCl solutions (0–1.0 M) at a flow rate of 60 mL/h. The obtained elute (10 mL/tube) was collected and the fractions were combined according to the carbohydrate content, as determined by the phenol–sulfuric acid method. A total of four homogenous fractions of CPSBS were obtained, named as PSBS-1, PSBS-2, PSBS-3 and PSBS-4, according to the order of elution.

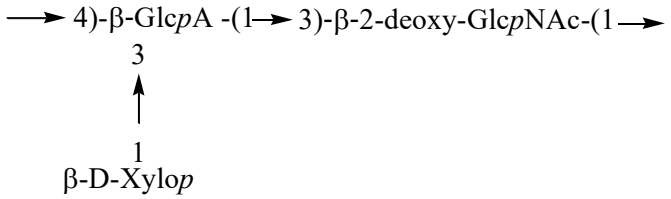
7.1.3. Composition analysis of the oligosaccharide fractions

Four fractions of CBSPS, which were named as PSBS-1, PSBS-2, PSBS-3, PSBS-4 were obtained subsequent to elution with distilled water and with increasing gradient of NaCl (0–1.0 M). . The crude polysaccharide (CPSBS) contained 65.86 % of total sugar and 42.55 % uronic acid, whereas the protein and sulfate contents were 8.30 % and 13.8 %, respectively. The results showed high carbohydrate contents for all these fractions. However, carbohydrate content of PSBS-2 was relatively higher than other fractions. Further monosaccharide composition analysis by the HPLC method specified PSBS-2 had a simpler composition consisting of glucose derivatives and xylose. The amount of sulphate was almost non-detectable (ND) in the fraction PSBS-2 with a trace quantity of protein (0.05 %).

7.1.4. Structural Characteristics of BSPSQ2

The sequence of the purified polysaccharide was determined by a combination of composition analysis, IR and NMR. UV–vis spectra of BSPS-2 fractions were acquired using Varian Cary 50 UV-visible spectrophotometer (Varian Cary, USA) which showed no optical absorption between 200 to 400 nm, suggesting the absence of protein and nucleic acids. The fraction PSBS-2 showed IR absorption at 914 cm^{-1}, which was characteristic of β-glycosidic linkages. The structural features of the purified polysaccharide fraction PSBS-2

were elucidated by Comprehensive nuclear magnetic resonance (NMR) techniques encircling DEPT analysis (distortionless enhancement by polarization transfer), ^{1}H-^{1}H COSY (homonuclear correlation spectroscopy), HSQC (heteronuclear single-quantum correlation), HMBC (heteronuclear multiple bond correlation), and nuclear overhauser effect (NOE) spectroscopy experiments. These results suggested that the following linkage sequence of glycoside residues were existing in PSBS-2.



Since the structure of the main chain of the polysaccharide was matching with the repeating unit of the nonsulphated glycosaminoglycan hyaluronan, we inferred it would be appropriate to consider it as a xylated hyaluronate.

7.3. Antioxidant, antidiabetic and anti-inflammatory activities of the polysaccharides isolated from *B. spirata*

The purified glycosaminoglycan reported significantly greater primary antioxidant potentials by inhibiting the model free radicals $ABTS^{+}$ and DPPH (IC_{50} 0.67 and 0.56 mg/mL, respectively) when compared to the other fractions as well as the standard, α-tocopherol (IC_{50} 0.73 and 0.60 mg/mL, respectively) ($p < 0.05$). The glycosaminoglycan-xylopyranan displayed potential inhibitory activities (IC_{50} 0.36 mg/mL) against pro-inflammatory enzyme 5-LOX when compared to the standard (IC_{50} 0.93 mg/mL). Compared to the other fractions isolated out of the crude polysaccharide, the purified glycosaminoglycan-xylopyranan demonstrated better ability to deter the enzymes α-glucosidase and α-amylase (IC_{50} 0.58 and 0.55 mg/mL, respectively).

CHAPTER 8: SUMMARY

A summary of complete study and discussion of the new research findings from the present work were detailed in this chapter.

REFERENCES

The references from all the chapters have been cited in this section.

PUBLICATIONS

The list of published and accepted publications (two in numbers) from the thesis work is detailed in this section.

Peer-reviewed publications:

1) **Soumya Salas[1]**, Kajal Chakraborty[1] (2018). First report of bioactive sterols from the muricid gastropod *Chicoreus ramosus*. Steroids. 137: 57-63

2) **Soumya Salas[1]**, Kajal Chakraborty[1] (2018) An unreported polyether macrocyclic lactone with antioxidative and anti-lipoxygenase activities from the Babylonidae gastropod mollusc *Babylonia spirata*. Medicinal Chemistry Research. 27: 2446. https://doi.org/10.1007/s00044-018-2248-z

3) Kajal Chakraborty[1], **Soumya Salas[1]**, Minju Joy[2] (2018) An unreported bis-abeo cembrane-type diterpenoid with antioxidative and anti-lipoxygenase activities from the muricid gastropod mollusc *Chicoreus ramosus*. Natural Product Research. *22: 1-9*

4) **Soumya Salas[1]**, Kajal Chakraborty, P. T. Sarada and P. Vijayagopal (2018) Nutritional composition of the branched murex *Chicoreus ramosus* (Linnaeus, 1758) (Family: Muricidae). Indian Journal of Fisheries. 65 (4):102-108. ISSN: 09706011

5) Kajal Chakraborty[1], Minju Joy[1], **Soumya Salas[1]** (2019) First report of a lactonic disecosteroid from the buccinid gastropod *Babylonia spirata*. Steroids, 143: 41-48

6) Kajal Chakraborty & **Soumya Salas** (2019): First report of antioxidant 1H-benzochromenone from muricid gastropod *Chicoreus ramosus* as dual inhibitors of pro-inflammatory 5-lipoxygenase and carbolytic enzymes, Natural Product Research, pp. 1-10. doi:10.1080/14786419.2019.1647428.

7) Kajal Chakraborty & **Soumya Salas** (2019): Antioxidant drimane type sesquiterpenoid from muricid gastropod *Chicoreus ramosus* attenuates pro-inflammatory 5-lipoxygenase and carbolytic enzymes. Journal of Food Biochemistry 10.1111/jfbc.12991.(In press)

8) Kajal Chakraborty & **Soumya Salas** (2019): First report of a glycosaminoglycan-xylopyranan from the buccinid gastropod mollusk *Babylonia spirata* attenuating pro-inflammatory 5-lipoxygenase. Journal of Food Biochemistry (Accepted manuscript)

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